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Original Research

Comparison of the effects of levetiracetam and valproic acid on neural tube defect formation in the chick embryo: an experimental study

Neural tube defect

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Abstract

Aim: The aim of this study is to compare the dosage-related effects of levetiracetam and valproic acid on neural tube defect formation in chick embryos, when applied as monotherapy and in combination. Material and Method: A total of 360 fertilized pathogens-free white-Leghorn eggs were used in the study. The eggs were allowed to incubate for 72 hours. Total of six groups were formed where each medication was administered in low (250 mcg) and high (500 mcg) doses and in combination. Results obtained from the control and sham groups were compared. Results: The prevalence of NTD was found to be significantly lower in the group that received levetiracetam compared to the group that was treated with valproic acid. It was determined that NTD prevalence increased with a dosage increase in both groups. The prevalence of NTD was found to be significantly higher in groups where the two medications were administered in combination compared to the groups that received a single medication. Discussion: Both levetiracetam and valproic acid have the potential to create NTD. Valproic acid has a higher potential of creating NTD compared to levetiracetam. The likeliness of causing NTD significantly increases depending on dosage for both medications. Both medications have the potential to create NTD during pregnancy and must be used with caution.

Keywords

Neural Tube Defect; Levetiracetam; Valproic Acid; Teratogenity

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Introduction

Central nervous system anomalies are among the most prevalent anomalies [1,2]. The most common anomalies within this group are those of neural tube formation. Certain factors are known to be involved in the etiology of neural tube formation anomalies. The fact that the same anomalies have been detected in embryos that were not exposed to these known factors suggests the existence of other factors involved in the etiology [1,3].

Folic acid deficiency is one of the most prominent environmental factors in the etiology of NTD formation [3], and it could be suggested that the folic acid metabolism is in turn affected by the environmental factors that commonly result in NTD. The use of anti-epileptic medications during pregnancy is also known to have a role in the development of congenital anomalies [4].

Many antiepileptics that are commonly used today are considered teratogens and it is recommended to use antiepileptics either at lower doses during pregnancy or substituted by other medications [5,6].

In this experimental study, the teratogenic effects of levatiracetam and valproic acid during pregnancy when anti-epileptic medications are required have been comparatively evaluated in the chick embryo. It was investigated whether the development of neural tube defects was associated with the dosage of levatiracetam and valproic acid used in the study (dosage comparison). The effects of combined levatiracetam and valproic acid therapy on NTD prevalence was evaluated in comparison to monotherapy.

Material and Method

Total of 360 specific pathogen-free (SPF), zero- day fertile, white-Leghorn eggs were obtained from the Bornova Veterinary Control Institute under cold chain for the purpose of comparative analysis of the effects of levetiracetam and valproic acid in terms of causing NTD in the chick embryo.

The eggs, which were obtained at different periods, were distributed into 3 main groups of 100 eggs each, 1 control group with 50 eggs, and 1 sham group with 10 eggs. Each of the three main groups with 100 eggs was evaluated under two subgroups with 50 eggs, where normal and high doses of medications were administered. Each egg was numbered and the obtained results were recorded (Table 1).

Parenteral forms of levatiracetam (Keppra) and valproic acid (Depakine) were obtained to be injected into the eggs. Each medication was diluted with physiological serum and the normal and high doses were ensured to be of an equal volume of 0,1 ml. Medication concentrations of 250 mcg were used as the normal dose, and 500 mcg as high dose.

The site of injection was cleansed with alcohol and Betadine prior to injection. The prepared medications were developed with physiological serum in appropriate concentrations and homogenization was ensured by centrifugation. Medication material (0,1 ml) was injected under the embryonic disc in the egg with a 31- gauge needle. In order to prevent contamination at the injection site, this area was sealed with paraffin. Each egg prepared to be incubated was kept under cold chain again, ensuring that the development of the embryos would be suppressed by the outer temperature and the eggs would begin incubation simultaneously. After all eggs were prepared, they were incubated in a Cimuka brand incubator at 37.8 °C and 60-70% relative humidity. Each egg in the incubator was rotated at 6- hour intervals so that the embryo material would not stick to different tissues within the egg. The eggs were left to incubate for 72 hours and were allowed to reach stage 18-20 according to the Hamburger-Hamilton stages. At the end of 72 hours, all eggs were removed from the incubator and were introduced to cold chain in order to suppress their development due to the effect of the outer temperature.

Table 1. NTD detection rates were compared in percentages across groups depending on dosage.

	Total Eggs	Number of Embryos Survived	Number of NTD Percentage
Group 1a LVT 250 mcg	50	46 92,0%	5 10,9%
Group 1b LVT 500 mcg	50	45 90,0%	5 15,6%
Group 2a VA 250 mcg	50	40 80,0%	10 25,0%
Group 2b VA 500 mcg	50	40 80,0%	12 30,0%
Group 3a LVT+VA 250mcg+250mcg	50	43 86,0%	11 25,6%
Group 3b LVT+VA 500mcg+500mcg	50	39 78,0%	14 35,9%
Control Group 0,1ml PS	50	47 94,0%	0 0,0%
Sham	10	9 90,0%	0 0,0%
Total	360		

As the eggs were being removed from the incubator, they were cracked open in laboratory conditions and the embryo materials were revealed. The embryos were isolated from the surrounding tissues and were investigated under loop with 4x magnification in a petri dish; other tissues attached to the embryo were removed with microforceps (Figure 1). Each embryo was then transferred and preserved in containers that contained 10% formol for the purpose of carrying out microscopic assessments later on.

Whole embryos that were not yet sectioned were also evaluated under a microscope with various lighting effects to obtain better images. Whole embryos that were evaluated were embedded in paraffin blocks and 5-micron sections were taken from the regions that manifested NTD. The sections that were stained with hematoxylin-eosin, were inspected under microscope at various levels of magnification (Figure 2,3). The sections were investigated under the Olympus BX61 brand microscope after staining and their images were captured.

(Ethics committee approval was obtained from Istanbul University Animal Research Local Ethics Committee: 23/07/2009-99/90)

Statistical Analysis

The number of embryos obtained at the end of the experiment and the presence of neural tubes was recorded in the Excel software. A chi-square test and Fisher's exact test (two-tailed; nonparametric) were used to assess the differences between the groups with respect to major developmental anomalies. A p-value < 0.05 was considered statistically significant.

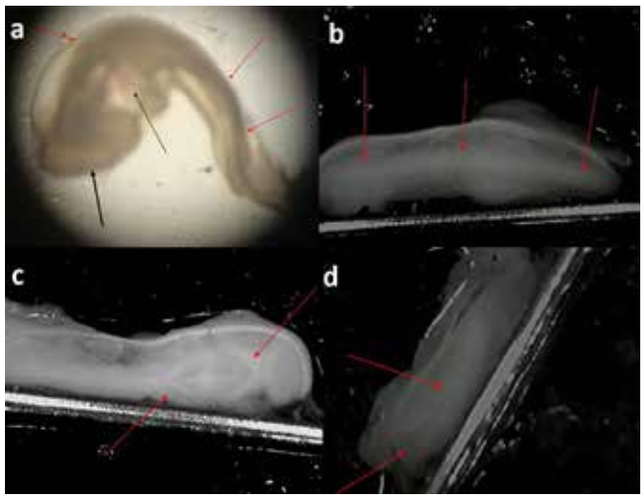

Figure 1. Image from the evaluation of major anatomical structures in the embryo under loop. Heart sac (black arrow), vertebral integrity (red arrows), eyeball (bold black arrow), somites (red arrows) are visible (a). Dorsal view image of the embryo; it can be seen that the neural groove is completely closed (b). Red arrows indicate that the neural groove is not closed in the tail region of the embryo (c,d).

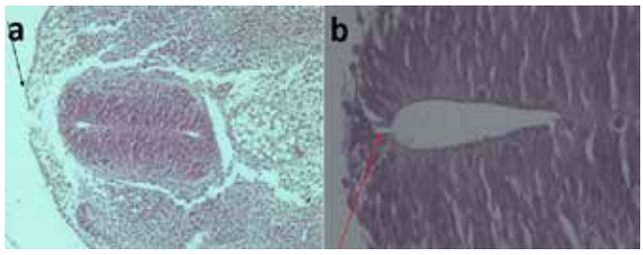

Figure 2. One of the image of a hematoxylin-stained embryo selected from the group treated with 250 mcg levetiracetam (Group 1a). It is shown that the neural groove is closed. There is no irregularity in the arrangement of the cells proliferated during the closure of the neural groove.

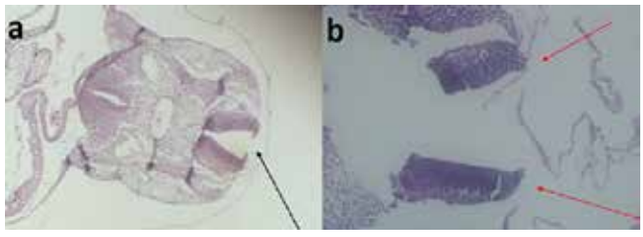

Figure 3. The image of a hematoxylin-stained embryo selected from the group treated with 500 mcg levetiracetam (Group 1b). It is shown that the neural groove is open under 4x magnification (a). Proliferation in cells surrounding the neural groove is not regular across all regions under 20x magnification (b).

Results

It was determined that embryo loss increased as drug concentration increased in all groups, regardless of whether the drugs were administered separately or in combination. Moreover, the prevalence of NTD was also found to increase depending on the dose in all groups (Table 1).

A total of 46 embryos were obtained from the eggs evaluated within Group 1a, which received 250 mcg levetiracetam. No embryos were found in 4 eggs. NTD was detected in 5 of the 46 obtained embryos. Fifty eggs evaluated within Group 1b were administered 500 mcg levetiracetam. Forty-five embryos were obtained in total. No embryos were found in 5 eggs. NTD was detected in 7 of the 45 obtained embryos.

Valproic acid (250 mcg) was administered to the 50 eggs evaluated within Group 2a. A total of 40 embryos were obtained. No embryos were found in 10 eggs. NTD was detected in 10 of the 40 obtained embryos. Valproic acid (500 mcg) was administered to the 50 eggs evaluated within Group 2b. Forty embryos were obtained in total. No embryos were found in 10 eggs. NTD was detected in 12 of the 40 obtained embryos.

Fifty eggs evaluated within Group 3a were administered with 250 mcg levetiracetam and valproic acid. Forty-three embryos were obtained in total. No embryos were found in 7 eggs. NTD was detected in 11 of the 43 obtained embryos. Fifty eggs evaluated within Group 3a were administered with 500 mcg levetiracetam and valproic acid. Thirty-nine embryos were obtained in total. No embryos were found in 11 eggs. NTD was detected in 14 of the 39 obtained embryos.

Fifty eggs evaluated within the control group (Group 4) were administered with 0,1 cc physiological serum. Forty-seven embryos were obtained in total. No embryos were found in 3 eggs. No NTDs were detected in the 47 obtained embryos. No drugs were administered to the 10 eggs evaluated within the sham group. These eggs underwent all experimental procedures including injections. Ten embryos were obtained in total. No NTDs were detected in the obtained embryos.

The comparison of results from Group 1a and Group 2b, which were treated with levetiracetam, revealed that the prevalence of neural tube defects was significantly higher in the group that received a higher dose of the drug (Group 1b) ($p = 0.007$). Similarly, the comparison of results from Group 2a and Group 2b in Group 2, which was treated with valproic acid, revealed that the prevalence of neural tube defects was significantly higher in the group that received a higher dose of the drug (Group 2b) ($p = 0.00001$).

In Group 3, we investigated the effect of administering combined levetiracetam and valproic acid on neural tube defect formation in the chick embryo. The comparison of results from Group 3a, which was the group that received low-dose medication, and Group 3b, which was the group that received high-dose medication, revealed that the prevalence of neural tube defects was significantly higher in Group 3b; the group that received high-dose medication ($p = 0.00001$).

The comparison of the group that was administered normal-dose levetiracetam (Group 1a) with the group that received normal-dose levetiracetam and valproic acid in combination (Group-3a) revealed that the prevalence of neural tube defects was significantly higher in the group that received both medications ($p = 0.007$).

The comparison of the group that was administered high-dose levetiracetam (Group 1b) with the group that received levetiracetam and valproic acid in combination (Group 3b) revealed that the prevalence of neural tube defects was significantly higher in the group that received both medications ($p = 0.00001$). The prevalence of neural tube defects was found to be significantly higher in groups that were administered with levetiracetam and valproic acid when compared to the control group.

It was determined that the prevalence of NTD and embryo loss increased in all groups at higher doses of the medications (Figure 4-7).

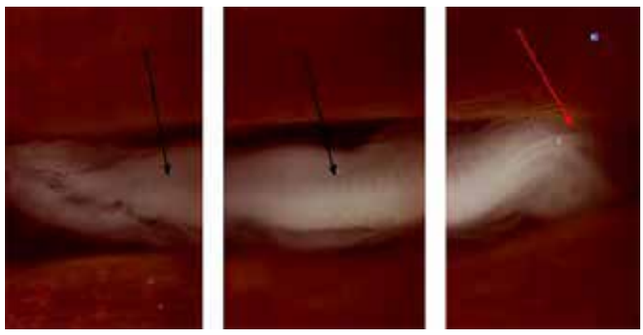

Figure 4. The three figures respectively present the head, trunk, and tail regions of the embryo under the microscope with the fluorescence effect. The photographs were captured under different focuses by adjusting the micrometer screw setting. It is shown that the neural groove is closed at the neck and trunk and that the neural groove is not closed in the tail region of the embryo.

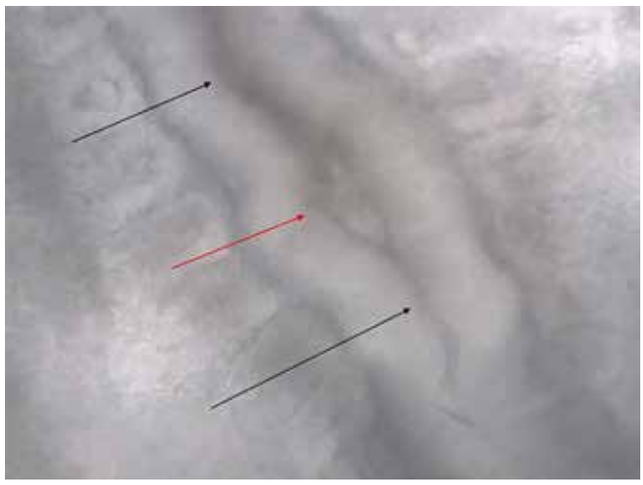

Figure 5. Coronal section showing that the neural groove is not completely closed at the tail region. (40x magnification)

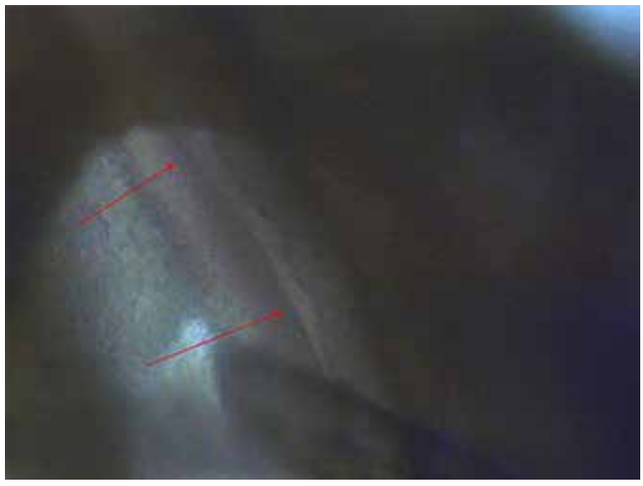

Figure 6. Coronal section showing that the neural groove is not completely closed at the tail region. (40x magnification) Captured under flurosence effect.

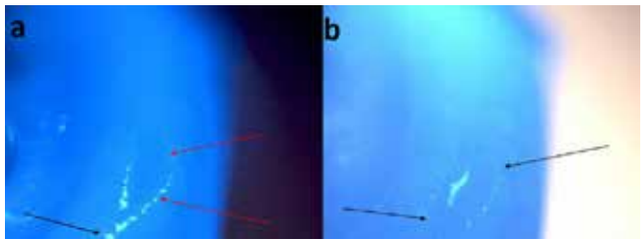

Figure 7. Coronal section showing that the neural groove is not completely closed at the tail region (red and black arrows) (a). Captured using blue light.

Discussion

The old generation medications used today as the first line treatments still make up the primary step of anti-epileptic treatment despite their known toxic and teratogenic effects [1,4,7,8]. There has been an increasing tendency to prefer new generation anti-epileptic medications over this group of medications referred to as the old generation anti-epileptics [8]. Numerous retrospective and prospective clinical studies have tried to reveal the mechanisms underlying the toxic and teratogenic effects of older generation anti-epileptics and the rates at which these effects are manifested [7]. Information regarding the levels and rates of similar effects associated with new generation anti-epileptics are scarce in the literature.

Valproic acid is among medications with established teratogenic effects when used during pregnancy [9]. Associated congenital anomalies, including NTD have been shown in many retrospective and prospective studies [10,11]. A study by G.L.Barnes et al. investigated the relationship between the teratogenic effect of VA on somites in the chick embryo and PaxC1 gene expression [12]. In this study, various doses of VA were administered, and it was concluded that the survival rate of the embryos decreased as the dose increased.

Among older generation anti-epileptics, carbamazepine, and phenytoin, although at a relatively low rate, have been shown to cause neural defects [9,13]. The fact that administering these two medications at the same dosage resulted in different rates of neural tube defect suggests that the affected biochemical paths are different [8]. Therefore, it would make sense to consider while trying to reveal the mechanism underlying any congenital anomaly, particularly those that appear during the embryonic stage, that it is not a single biochemical mechanism that is affected but the primary morphological processes that initiate fetal development. First of all, it must be considered that the teratogenic effect brought about by the use of anti-epileptics or any other teratogens in the first trimester would affect the differentiation of the endoderm, mesoderm, and ectoderm embryonic germ layers.

The most well-known external factor that result in a high prevalence of neural tube defect is a folic acid deficiency [14,15]. For this reason, the teratogenic agents that cause neural tube defect are believed to affect the folic acid metabolism. However, the fact that the known teratogenic agents manifest this teratogenic effect of varying levels suggests that these agents either affect the folic acid mechanism at varying levels are also affected different biochemical paths.

Similar studies done previously also have shown that levetiracetam causes NTD [16,17]. In the experimental study conducted by Ozer et al. on chick embryos, it was shown that levetiracetam led to midline defects and a reduction in cell proliferation [16]. Ozgural et al. reported that the concentration of calcium ions were affected and NTD prevalence increased depending on the dosage; and emphasized that genetic and developmental factors also needed to be considered alongside teratogenic effects [17]. The same effects were found to appear via the Fibroblast Growth Factor-2 in a study conducted by Duransoy et al [18]. This study, on the other hand, compared valproic acid, which is known to have a high potential for causing NTD, and levetiracetam, the effects of which are not as well-known, and allowed

comparison of the risk potentials of these two anti-epileptics in terms of causing NTD.

In this study, although levetiracetam presented a weaker teratogenic effect in terms of causing neural tube defect when compared to valproic acid, its teratogenic effect was significantly higher in comparison to the control group. Moreover, it was determined that survival decreased and the prevalence of neural tube defect increased with an increasing dose in eggs injected both with levetiracetam and valproic acid. Similarly, survival rates associated with the combination of the two medications were lower compared to the groups that were administered a single medication, and neural tube defects were more prevalent in the case of combined use.

It was concluded that levetiracetam, despite resulting in higher survival and a lower prevalence of neural tube defect than valproic acid, had a significantly higher teratogenic effect when compared to the control group. The combination of the two medications has a potential negative effect on NTD prevalence and survival.

Conclusion

In our experimental study, we intended to reveal the neural tube defect rates associated with levetiracetam compared to valproic acid. Both medications were applied at equal doses across groups and the results were evaluated by comparing. Both medications were evaluated statistically in terms of causing NTD in chick embryos in comparison to the control group and the results were significant. Furthermore, the higher prevalence of NTD resulting from the combined use of the two medications compared to the NTD rate associated with their use as monotherapy was considered statistically significant. It was concluded that, when used in combination, LVT and VA increased each other's teratogenic effect in the etiology of NTD.

Scientific Responsibility Statement

The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement

All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest

None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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